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APPLICATION

FOR

UNITED STATES LETTERS PATENT

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TITLE: West Nile Virus Vaccine

WEST NILE VIRUS VACCINE

Cross-Reference to Related Applications

This application claims priority from U.S. Serial No. 60/426,592, filed November 15, 2002, which is incorporated by reference herein in its entirety.

Field of the Invention

This invention relates to vaccines against West Nile virus.

Background of the Invention

Since its earliest detection in the northern hemisphere, West Nile (WN) virus has continued to spread rapidly across North America. The first cases were diagnosed in the New York area in 1999, and by 2002 human mortality increased to over 150 cases and the virus spread has continued, reaching as far as California. The appearance of infected/dead birds indicates that there is a large pool of infected mosquitoes in the geographical areas of incidence. To date, there is no effective drug treatment against West Nile virus and methods of surveillance and prevention are not significantly impacting the number of cases of human infection. Thus, the risks of the virus migrating into the southern American continent, as well as an epidemic in underdeveloped countries, are extremely high.

West Nile virus is a member of the flavivirus family. These viruses are small, enveloped, positive-strand RNA viruses that are of concern in many medical and veterinary settings throughout the world. Examples of flaviviruses in addition to West Nile virus include Yellow Fever virus, Japanese Encephalitis virus, and Dengue viruses.

Flavivirus proteins are produced by translation of a single, long open reading frame to generate a polyprotein, which undergoes a complex series of post-translational proteolytic cleavages by a combination of host and viral proteases to generate mature viral proteins (Amberg et al., J. Virol. 73:8083-8094, 1999; Rice, "Flaviviridae," In *Virology*, Fields (ed.), Raven-Lippincott, New York, 1995, Volume I, p. 937). The virus structural proteins are arranged in the polyprotein in the order C-prM-E, where "C" is capsid, "prM" (or "pre-membrane") is a precursor of the viral envelope-bound M (membrane) protein, and "E" is the envelope protein.

These proteins are present in the N-terminal region of the polyprotein, while the non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are located in the C-terminal region of the polyprotein.

Summary of the Invention

The invention provides nucleic acid molecules that include sequences encoding the pre-membrane and envelope proteins of West Nile virus and the capsid and non-structural proteins of Yellow Fever virus. The West Nile virus pre-membrane or envelope proteins of these chimeras include one or more attenuating mutations, which can be, for example, an amino acid substitution at positions 107, 316, and/or 440 of the envelope protein. As specific examples, the amino acid substitution at position 107 can be leucine to phenylalanine (or a conservative amino acid thereof); the amino acid substitution at position 316 can be alanine to valine (or a conservative amino acid thereof); and the amino acid substitution at position 440 can be lysine to arginine (or a conservative amino acid thereof).

The invention also includes chimeric flaviviruses encoded by the nucleic acid molecules described herein, as well as methods of inducing an immune response to West Nile virus in a subject by administration of such chimeric flaviviruses. Further, the invention includes the use of such chimeric flaviviruses in vaccination methods and their use in methods for preparing medicaments for use in such methods. The methods described herein can be carried out with subjects that are at risk of developing, but do not have, West Nile virus infection, as well as with subjects that are infected with West Nile virus. The invention also provides methods of making the chimeric flaviviruses described herein.

The invention provides several advantages. For example, as is discussed in more detail below, the attenuating mutations of the viruses of the invention result in decreased neurovirulence, yet do not adversely impact the ability of the viruses to induce an effective immune response. Thus, the viruses of the invention provide an effective and safe approach to preventing and treating West Nile virus infection.

Other features and advantages of the invention will be apparent from the following detailed description and the claims.

Brief Description of the Drawing

Figure 1 is a graph showing reciprocal neutralizing antibody titer ($PRNT_{50}$) results from ICR mice vaccinated with YF/WN *wt* or YF/WN_{FVR}. Individual neutralizing antibody titers against YF/WN *wt* virus are shown. White symbols denote mice that did not survive IP challenge with WNV NY-99 strain. 100% survival was obtained in groups vaccinated with 10^3 pfu dose of YF/WN *wt* and with 10^5 pfu dose of YF/WN_{FVR}. Only 40% mice survived challenge in the group vaccinated with 10^3 pfu of YF/WN_{FVR}.

Detailed Description

The invention provides vaccines and methods for use in preventing and treating West Nile (WN) virus infection. The methods of the invention involve vaccination of subjects with a live, attenuated chimeric flavivirus that consists of a Yellow Fever virus in which the pre-membrane and envelope proteins have been replaced with those of West Nile virus. The West Nile virus proteins of the chimeras of the invention include one or more attenuating mutations, as is described further below.

General methods for constructing and administering chimeric flaviviruses that can be used in the present invention are described in detail, for example, in U.S. patent application serial numbers 09/007,664, 09/121,587, and 09/452,638; International applications PCT/US98/03894 (WO 98/37911) and PCT/US00/32821 (WO 01/39802); and Chambers et al., J. Virol. 73:3095-3101, 1999, which are each incorporated by reference herein in their entirety. As is discussed further below, these methods are modified for use in the present invention by including a step of introducing one or more attenuating mutations into inserted West Nile virus sequences. Methods that can be used for producing viruses in the invention are also described in PCT/US03/01319 (WO 03/060088 A2), which is also incorporated herein by reference.

In one example of a chimeric virus of the invention, the attenuating mutation is in the region of position 107, 316, or 440 (or a combination thereof) of the West Nile virus envelope protein. The mutations can thus be, for example, in one or more of amino acids 102-112, 311-321, and/or 435-445 of the West Nile envelope protein. As a specific example, using the sequence of West Nile virus strain NY99-flamingo 382-99 (GenBank Accession Number AF196835) as a reference, lysine at position 107 can be replaced with phenylalanine, alanine at position 316 can be replaced with valine, and/or lysine at position 440 can be replaced with

arginine. In addition to the amino acids noted above, the substitutions can be made with other amino acids, such as amino acids that would result in a conservative change from those noted above. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, and leucine; aspartic acid, glutamic acid, asparagine, and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine. In a specific example, a chimera of the invention includes each of the specific substitutions noted above. Further, as is discussed further below, additional residues (e.g., positions 138, 176, and/or 280) can also be altered in the chimeric viruses of the present invention.

The vaccines of the invention can be administered in amounts and by using methods that can readily be determined by persons of ordinary skill in this art. The vaccines can be administered and formulated, for example, as a fluid harvested from cell cultures infected with the chimeric virus. The live, attenuated chimeric virus can be formulated as a sterile aqueous solution containing between 10^2 and 10^8, e.g., between 10^6 and 10^7, infectious units (e.g., plaque-forming units (pfu) or tissue culture infectious doses) in a dose volume of 0.1 to 1.0 ml, to be administered by, for example, subcutaneous, intramuscular, or intradermal routes. In addition, a mucosal route, such as the oral route, can be selected. Selection of an appropriate amount of chimera to administer can be determined by those of skill in this art, and this amount can vary due to numerous factors, e.g., the size and general health of the subject to whom the chimera is to be administered. The subject can be vaccinated a single time or, if necessary, follow-up immunization can take place.

As is noted above, the vaccines can be administered as primary prophylactic agents to a subject that is at risk of West Nile virus infection. The vaccines can also be used as secondary agents for treating West Nile virus-infected subjects by stimulating an immune response against the infecting virus. Also, although not required, adjuvants can be used to enhance the immunogenicity of the chimeric West Nile virus vaccines. Selection of appropriate adjuvants can readily be carried out by those of skill in this art.

The invention is based, in part, on the following experimental results.

Experimental Results

To increase the safety profile of a West Nile/Yellow Fever chimeric vaccine, we investigated whether attenuating point mutations in the envelope protein would reduce

neurovirulence. As is described in detail below, we constructed a YF/WN chimera that lacks the mouse neuroinvasiveness of West Nile virus and is less neurovirulent than the Yellow Fever vaccine strain YF 17D in both mice and monkey models. The following is a description of the envelope protein mutagenesis, and an assessment of safety, immunogenicity, and efficacy of this and related viruses in mouse and rhesus models.

Materials and Methods

YF/WN chimeric constructs and molecular procedures

Chimeric flaviviruses are constructed using the ChimeriVax™ technology, which involves using a two-plasmid system that was previously described (see, e.g., U.S. Patent Application Serial Nos. 09/007,664, 09/121,587, and 09/452,638; International applications PCT/US98/03894 (WO 98/37911) and PCT/US00/32821 (WO 01/39802); and Chambers et al., J. Virol. 73:3095-3101, 1999). The two-plasmid system provides plasmid stability in *E. coli* and a suitable method to manipulate the cloned yellow fever (YF) backbone, facilitating replacement of the YF prM and E genes with those of a flavivirus target. The West Nile (WN) virus prM and E genes used were cloned from WNV flamingo isolate 383-99, sequence GenBank accession number AF196835. Virus prME cDNA was obtained by RT-PCR (XL-PCR Kit, Perkin Elmer). The 5' end of WN prM gene was cloned precisely at the 3'end of the YF 17D capsid gene by overlap-extension PCR using Pwo polymerase (Roche). This cloning step maintains the integrity of the cleavage/processing signal encoded at the 3' end of the YF capsid gene. The 3' end of the E gene was also cloned precisely at the 5'end of the YF NS1 coding sequence by overlap-extension PCR. Use of this two-plasmid system in the cloning of the prME region of West Nile virus into the YF 17D backbone was described previously (Arroyo et al., Trends in Molecular Medicine 7(8):350-354, 2001). Silent mutations were introduced into the sequence of prM and E to create unique restriction sites *Bsp* EI and *Eag* I. Digestion of the two plasmids with these enzymes generated DNA fragments that were gel purified and ligated *in vitro* to produce a full-length chimeric cDNA. The cDNA was linearized with *Xho* I to facilitate *in vitro* transcription by SP6 polymerase (Epicentre). The RNA product was introduced into eukaryotic cell lines permissive for viral RNA translation and replication of the virus.

Point mutations were introduced at various E gene codons to produce variants of the original chimera coding for wild-type WN prME. Table 1 shows the mutation target sites and

the oligonucleotide sequences used to create the YF/WN chimeras described below. Site mutations were confirmed by sequencing of the envelope proteins (prME region) of the resulting viruses. Virus cDNA templates for sequencing originated from RNA extracted from virus supernatants (Trizol LS, Invitrogen), followed by RT-PCR (XL-PCR Kit, Perkin Elmer) and sequencing with the use of synthetic primers (Invitrogen) and a CEQ 2000 sequencer (Beckman).

Viruses and cell lines

Chimeric YF/WN (i.e., ChimeriVax™-West Nile) viruses were prepared by RNA transfection (passage 1 virus, P1) into a Vero E6 cell line (ATCC, CIDVR UMASS Medical Center Worcester, MA). Research master seeds (RMS) were prepared by additional amplifications (either passage 2 or 3 at a 0.001 MOI) in Vero E6 cells. Vero E6 cells were maintained in MEM (Invitrogen), 10% FBS (Hyclone). Preparation of preMaster Seeds (PMS) for manufacture of the vaccine was initiated by RNA transfection into a serum free Vero (SF-Vero) cell line (ATCC, Baxter/Immuno Orth, Austria), followed by an amplification passage in the same SF-Vero cell line to produce a P2 PMS or preMaster Seed. The SF-Vero cell line was propagated and maintained in a serum free, protein free media formulation VT-Media (Baxter/Immuno, Austria). The wild type WN virus used was a NY-99 strain (NY99-35262-11 flamingo isolate) obtained from CDC, Fort Collins, CO (CDC stock designation B82332W) with two additional passages in Vero E6 cells to produce a Master Virus Bank. YF 17D is a commercial vaccine (YF-VAX®, Aventis Pasteur, Swiftwater, PA) used here after reconstitution of the lyophilized product or after one passage in Vero E6 cells (ATCC, Acambis Inc., Cambridge, MA).

Mouse neuroinvasiveness and neurovirulence in adult and suckling mice; immunogenicity and challenge titer detection methods (inoculation procedure, plaque assay, PRNT)

Mice were inoculated intraperitoneally (IP) for neuroinvasion tests or during challenge with wild type WN virus. IP inoculation volumes were 100-200 µl administered with a 25G syringe. Adult and suckling mice were inoculated intracerebrally (IC) for neurovirulence testing. An inoculation volume of 20 µl administered on the right side of the frontal lobe of the brain was used for IC administration. Viruses were diluted in M199 with HEPES buffer (Invitrogen) and

20% FBS (Hyclone) unless otherwise indicated. Plaque assays in Vero cells were carried out to verify the titer of virus inoculi (Monath et al., J. Virol. 74(4):1742-1751, 2000).

Mice were observed for a period of 21 days to determine neuroinvasion, neurovirulence, or survival after West Nile virus challenge. Morbidity and mortality were observed/scored and survivors were euthanized.

To determine neutralizing antibody titers, mice were bled by the retroorbital route and serum was separated by centrifugation. Plaque reduction neutralization assays (PRNT) were used to measure titers of neutralizing antibody in serum.

Rhesus neurovirulence study

The test in rhesus monkeys to determine the neurovirulence of yellow fever (YF) vaccines, as described in WHO guidelines, was used to determine safety of YF/WN chimeras (Monath et al., J. Virol. 74(4):1742-1751, 2000). Animals were inoculated IC, and blood samples were obtained daily to measure viremia levels using the plaque assay technique. Animals were observed daily for signs of disease-associated symptoms, such as fever or tremors. Animals were euthanized 30 days after infection and brain and spinal chord tissues were then removed for histopathology. Neuropathology was scored following the WHO defined system and the values were analyzed for pathology relative to those of the YF 17D vaccine standard.

Rhesus immunogenicity and challenge

Rhesus monkeys were vaccinated by subcutaneous administration of a single 0.5 ml dose of vaccine containing a nominal 4 log10 PFU. Viremia was measured by plaque assays of the diluted serum on Vero cells using serum samples collected daily between days 0 to 10 (Monath et al., J. Virol. 74(4):1742-1751, 2000). Neutralizing antibody levels were measured by plaque reduction neutralization titer assays (PRNT) (Monath et al., J. Virol. 74(4):1742-1751, 2000). Animals were challenged 64 days post vaccination with wild-type WN virus NY99.

Genetic stability (*in vivo* and *in vitro* passage) and sequencing

A YF/WN *wt* (i.e., ChimeriVax™-WN_{01}) construct without attenuating mutations in the E protein was passed six times in Vero E6 cells followed by six passages in suckling mice by the IC route. YF/WN_{FVR} (i.e., ChimeriVax™-WN_{02}) preMaster Seed and Research Master Seed

constructs with 3 attenuating mutations introduced in the E protein were passed 12 and 10 times, respectively, in serum free, protein free SF-Vero cell substrate. All passages were performed with an initial 0.01 MOI followed by harvest on the third day and continuing without titration to determine virus potency. Virus titers used at each passage were later calculated by plaque assay. Neurovirulence of the passaged viruses was measured by adult or suckling mice inoculations IC. Viral RNA was then sequenced.

Results

Virulence phenotype of YF/WN *wt* relative to YF 17D (YF-VAX®)

The initial West Nile virus chimera encoded the envelope and premembrane protein genes of the WN NY99 wild type strain (i.e., YF/WN *wt* or ChimeriVax™-WN_{01}). This chimeric virus lacked the ability to cause encephalitis after IP inoculation at doses of 10^6 pfu in the ICR mouse (Table 2). Encephalitis was assessed by daily observation of changes in motor behavior, leading to paralysis and death. The YF/WN *wt* lack of neuroinvasion in the adult mouse was also observed in mice inoculated with the YF 17D vaccine by others (Ryman et al., Virology 230(2):376-380, 1997). In contrast, the WN NY99 wild type virus was lethal for mice when inoculated by the IP route with as little as 1-4 pfu (Beasley et al., Virology 296:17-23, 2002). The IC LD_{50} of YF/WN *wt* was estimated here to be between 10^3 and 10^5 pfu. The neurovirulence phenotype of YF/WN *wt* is lower than that of YF 17D virus, where the ICR mice IC LD_{50} is between 10^1 and 10^2 pfu. However, theYF/WN *wt* virus did not show a clear endpoint in the 21 day old mouse (Table 3).

Multi-site mutagenesis approaches

Amino acids in the envelope protein were changed to determine whether these changes would reduce the virulence of YF/WN chimeras. Changes in virulence were evaluated in the mouse model and compared to the neurovirulence of the original chimera YF/WN *wt*. Amino acid residues mapping to the YF/WN *wt* envelope (E) gene positions 107, 138, 176, and 280 were all mutated in a singular construct to encode amino acid residues F, K, V, and M, respectively. The new chimeric virus was identified as YF/WN_{FKVM}. Chimeras were then constructed in which each amino acid residue in the FKVM group was singly mutated to assess its individual role in neurovirulence (Table 4). In addition, mutations at amino acid residues 316

and 440 were mutated to V and R, respectively, based on previous data indicating that mutations in the E protein which mapped to these regions may function in the biology of the E protein third domain (Rey et al., Nature 375(6529): 291-298, 1995; Allison et al., J. Virol. 75(9):4268-4275, 2001). Our observations of the neurovirulence of chimeras having modified amino acids in the E protein indicated that residues 107, 316, and 440 are the most important amino acids contributing to neurovirulence of WN virus. Based on this information, a multi-site YF/WN_{FVR} construct was built and selected as our vaccine candidate. All multi-site chimeras grew to titers of 10^7 pfu/mL in serum free SF Vero cells.

Neurovirulence studies in mice and rhesus monkeys

Mouse neurovirulence of viruses with single or multi-site mutations in the envelope protein gene of YF/WN *wt* was measured in 21 day old mice inoculated by the IC route with virus doses between 10^4 and 10^5 pfu. This assessment identified residues 107 and 280 (Table 4) and the combination of 316/440 (Table 5) as the more dominant attenuating mutations as measured by mouse mortality and relative average survival time (AST). The chimera selected as our vaccine candidate that had a combination of mutations F, V, and R at residues 107, 316, and 440, respectively, was avirulent in the adult mouse (Table 6). However, some neurovirulence was observed in two day old suckling mice, similar to the ChimeriVax™-JE vaccine that is safe for humans (Monath et al., Vaccine 20:1004-1018, 2002).

The rhesus monkey neurovirulence phenotype of the West Nile chimeras was measured with the YF/WN *wt* construct and compared to that of the YF 17D vaccine. Rhesus monkeys were inoculated by the IC route with YF/WN *wt* and compared to YF 17D vaccine inoculates. The chimera induced viremia titers and duration similar to the YF 17D vaccine. This virus was not more neurovirulent than the YF vaccine, indicating that the YF/WN chimeras are as safe as the YF 17D vaccine.

Immunogenicity studies in mice and rhesus monkeys

We compared the immunogenicity of the YF/WN *wt* chimera and the YF/WN_{FVR} construct, which has mutations at envelope residues 107, 316, and 440. Mice were inoculated by the subcutaneous (SC) route with vaccine doses ranging between 2 to 5 $\log_{10}$ pfu. Serum was collected four weeks after immunization and titrated for neutralizing antibodies. The data in

Figure 1 show that site-targeted attenuation of the YF/WN *wt* construct resulted in a less immunogenic virus. Intraperitoneal challenge of the immunized mice with wild type WN NY99 virus revealed a vaccine potency-to-efficacy correlation with the constructs. All of the mice immunized with a 10^5 pfu/ml dose of either of the YF/WN chimeras were protected from virulent virus challenge.

In rhesus monkeys, the immunogenicity of vaccine candidates with one, two, or three attenuating mutations, YF/WN$_{107}$F, YF/WN, $_{316}$V$_{440}$R, or YF/WN$_{FVR}$ were equal. There were no significant differences in the respective antibody titers when the animals received a 10^4 pfu vaccine dose. Efficacy studies determined by post immunization challenge with wild type WN NY99 virus clearly showed in the WN vaccine candidates tested in the rhesus model were 100% efficacious relative to control animals (Table 7).

Post-vaccination viremia in rhesus macaques was of similar duration relative to that induced by the YF 17D vaccine, but lower in magnitude, with a linear correlation to the number of attenuating unique mutations in the chimeras (Table 8). Nevertheless, assessment of safety based on viscerotropism (or post vaccination viremia) shows that any one of the vaccine candidates tested in the macaque model is safer than YF 17D.

Post IC challenge of the immunized rhesus macaques with 5.38 $\log_{10}$ pfu of WN NY99 did not produce detectable viremia, and no clinical signs of disease or mortality were observed in animals vaccinated with YF/WN constructs. Significant viremia due to WNV replication was detected in the YF 17D vaccinees. Viremia levels detected in animals in this group had a mean $\log_{10}$ pfu titer of 2.25±0.62, with a mean duration of 3.5 days. These viremia levels are similar to those observed in the non-vaccinated control group (n=2), with a mean $\log_{10}$ pfu titer of 2.33±0.47 and 4.5 days mean duration. Two out of four rhesus macaques vaccinated with YF 17D survived IC challenge with WN NY99 strain. The survivors lost appetite and showed symptoms of lethargy with elevated temperatures post challenge. Animals that were euthanized post WNV NY99 strain challenge presented symptoms including fever and tremors.

Genetic stability

In vitro and *in vivo* substrate-passage studies with YF/WN *wt* or YF/WN$_{FVR}$ chimeras were conducted to determine genetic stability of the constructs when grown in stationary cultures. After six *in vitro* Vero E6 cell passages, followed by six *in vivo* ICR adult mouse brain

passages of YF/WN *wt*, we found no undesirable mutations in the envelope gene (prM, E) region of this chimera. A heterozygous mutation in the E protein at position E336, resulting in a cysteine to serine, was identified after ten *in vitro* passages of the YF/WN$_{FVR}$ virus in Vero E6 cells. In a separate study, *in vitro* passage of YF/WN$_{FVR}$ SF-Vero cells resulted in selection of a mutation at position E 313, which changed the amino acid at this position from glycine to arginine. During all of the serial passages of the virus in Vero cells, no reversions/mutations were detected at target residues 107F, 316V, or 440R, which are involved in virus neurovirulence.

Table 1

Switch Oligonucleotides used for site-mutagenesis

E Protein position and residue	Primer	Marker Site
107 L F	5' CAACggCTgCggA**TTTTTT**ggCAAAggATCCATTgACACATgCgCC 3'	*Bam* HI
138 E K	5' gAAAgAgAATATTAAgTAC**AA**AgTggCCATTTTTgTCC 3'	*Ssp* I
*176 V	5' gCCCTCgAgCggCCgATTCAgCATCACTCCTgCTgCgCCTTCA**g**TCACAC 3'	
*176 Y	5' gCCCTCgAgCggCCgATTCAgCATCAC-3'	
280 K M	5'-gCAACACTgTCA**T**gTTAACgTCgggTCATTTg 3'	*Hpa* I
316 A V	5'-CTTgggACTCCCg**T**ggACACCggTCACggCAC-3'	*Age* I
440 K R	5'-ggggTgTTCACTAgTgTTggg**C**gggCTgTCCATCAAgTg-3'	*Spe* I

Primers for site-directed mutagenesis to create mutant attenuated Yellow Fever/West Nile Virus. Nucleotide changes that introduce a new amino acid are indicated in bold. Silent restriction sites introduced are underlined. Primers indicated with an * (asterisk) are cloning primers used to sub-clone the fragment. One incorporates a nucleotide change while the other does not.

Table 2

Neuroinvasiveness of YF/WN *wt* (ChimeriVax™-West Nile 01) relative to YF 17D, dose response in ICR mice[1]

Virus IP	Dose ($\log_{10}$ PFU)	% Mortality (no. dead / no. tested)	AST[2]
YF/WN *wt* (P2)	0.89	0 (0/5)	-
	2.23	0 (0/5)	-
	3.24	0 (0/5)	-
	4.06	0 (0/5)	-
	5.45	0 (0/5)	-
	6.51	0 (0/5)	-
YF17D (ATCC)	2.78	0 (0/3)	-
	4.48	0 (0/3)	-
Sham	N/A	0 (0/3)	-

[1] Harlan-Sprague ICR strain 3-4 weeks old female mice were used.
[2] AST=Average Survival Time.
YF/WN *wt* P2 indicates a second-generation passage virus on Vero cells. West Nile virus strains are typically neuroinvasive after IP inoculation.

Table 3

Neurovirulence of YF/WN *wt* (ChimeriVax™-West Nile 01) relative to YF 17D, dose response (Harlan-Sprague ICR strain 3-4 weeks old female mice)

Virus IC	Dose[1] (log_{10} PFU)	% Mortality (no. dead / no. tested)	AST
YF/WN *wt* (P2)	-2	0 (0/5)	-
	-0.30	0 (0/5)	-
	0.89	20 (1/5)	11
	2.23	0 (0/5)	-
	3.24	20 (1/5)	10
	4.06	60 (3/5)	9
	5.45	20 (1/5)	9
YF17D (ATCC)	0	20 (1/5)	9
	0	60 (3/5)	10.3
	0.9	100 (5/5)	9.2
	0.98	100 (5/5)	8.2
	2.78	100 (5/5)	8
Sham	N/A	0 (0/3)	-

[1]Actual dose delivered intracerebrally (IC) assumed to be 20 μl for back titration calculations shown.

Table 4

Neurovirulence of ChimeriVax™-WN 01 site-directed mutagenesis variants at E protein residues 107, 138, 176, and 280 tested in adult mice[1]

Virus (Vero Passage)	Target Dose ($\log_{10}$ PFU)	Back Titration Dose ($\log_{10}$ PFU)	% Mortality (no. dead/no. tested)	AST
YF/WN *wt*	4	4.87	100 (5/5)	8.6
(P3)	5	6.09	60 (3/5)	9
YF/WN $_{107}$F	4	4.22	0 (0/5)	-
(P2)	4	4.42	0 (0/8)	-
	5	4.99	0 (0/5)	-
YF/WN $_{138}$K	4	4.26	60 (3/5)	10.33
(P3)	4	4.41	63 (5/8)	11.4
	5	5.48	60 (3/5)	9.33
YF/WN $_{176}$V	4	4.42	80 (4/5)	12.5
(P3)	5	5.54	80 (4/5)	11
YF/WN $_{280}$M	4	4.14	40 (2/5)	9
(P3)	4	4.55	89 (7/8)	11.86
	5	5.14	0 (0/5)	-
YF/ $_{107}$F$_{138}$K$_{280}$M	4	3.70	0 (0/5)	-
(P2)	5	4.81	0 (0/5)	-
YF/ $_{107}$F$_{138}$K$_{176}$V$_{280}$M	4	4.13	0 (0/5)	-
(P3)	5	5.10	20 (1/5)	7
YF-VAX	3	2.77	100 (5/5)	9
WN NY99	4	3.90	100 (5/5)	5

[1]Mice inoculated IC were Taconic ICR 3-4 week old females. P2 and P3 indicate second and third generation virus passage on Vero cells, respectively.

Table 5

Neurovirulence of ChimeriVax™-WN 01 site-directed mutagenesis variants at E protein residues 316 and 440 tested in adult mice[1]

Virus IC (Vero passage)	Back Titration Dose ($\log_{10}$ PFU)	% Mortality (no. dead/no. tested)	AST
YF/WN *wt* (P3)	4.11	83 (10/12)	9.2
	4.74	60 (3/5)	10.33
	4.83	100 (8/8)	10.63
YF/WN$_{316}$V (P3)	4.09	25 (3/12)	12.33
	4.67	38 (3/8)	10.67
	4.57	38 (9/24)	11.22
YF/WN$_{440}$R (P3)	4.17	83 (10/12)	9.22
	4.60	38 (3/8)	10.33
	4.35	56 (14/25)	11.21
YF/WN$_{316}$V$_{440}$R (P3)	3.9	17 (2/12)	16.5
	4.12	40 (2/5)	13
	3.71	36 (9/25)	12

[1] Mice inoculated IC were Taconic ICR 3-4 week old females. Results of three independent experiments are shown.

Table 6

Neurovirulence of ChimeriVax™-WN 02 ($YF/WN_{107}F_{316}V_{440}R$) for adult mice relative to YF/WN *wt*[1]

Virus IC (Vero passage)	Back Titration Dose (log_{10} PFU)	% Mortality (no. dead/no. tested)	AST
YF/WN wt (P2)	3.62	42 (5/12)	9.4
$YF/WN_{107}F_{316}V_{440}R$ (P4)	5.54	0 (0/12)	-
$YF/WN_{107}F_{316}V_{440}R$ (P4)	3.72	0 (0/12)	-

[1]Mice inoculated IC were Taconic ICR strain 3-4 week old females.

Table 7. Reciprocal neutralizing antibody titers ($PRNT_{50}$) against YF/WN *wt* virus. Rhesus monkeys were inoculated by the subcutaneous route with YF 17D or YF/WN vaccine candidates as specified.

Monkey	Vaccine	Dose Log_{10} PFU	Day postimmunization (dpi)			Day postchallenge (dpc)	
			14 dpi	30 dpi	63 dpi	15 dpc	31-34 dpc
M017	YF 17D	4.49	NT	NT	<40	-[c]	-
BI01	YF 17D	4.49	NT	NT	<40	>640	NT
R286	YF 17D	4.49	NT	NT	<40	-	-
T081	YF 17D	4.49	NT	NT	<40	>640	NT
N313	$YF/WN_{107}F$ (P2)	4.19	160	> 640	> 640	2560	5120
P367	$YF/WN_{107}F$ (P2)	4.19	< 40	640	640	5120	2560
T087	$YF/WN_{107}F$ (P2)	4.19	< 40	640	> 640	2560	1280
AE81	$YF/WN_{107}F$ (P2)	4.19	< 40	> 640	160	> 10240	> 20480
GMT[a]			**57**	**640**	**453**	**4305**	**4305**
R918	$YF/WN_{316}V_{440}R$ (P3)	4.0	< 40	320	> 640	> 1280	2560
N577	$YF/WN_{316}V_{440}R$ (P3)	4.0	< 40	> 160	320	> 1280	2560
M233	$YF/WN_{316}V_{440}R$ (P3)	4.0	< 40	> 160	320	640	1280
T757	$YF/WN_{316}V_{440}R$ (P3)	4.0	< 40	40	> 640	> 1280	> 5120
GMT			**40**	**135**	**453**	**1076**	**2560**
J729	YF/WN_{FVR} (P4)	3.92	< 40	320	80	> 5120	> 5120
T445	YF/WN_{FVR} (P4)	3.92	80	640	160	640	> 5120
T086	YF/WN_{FVR} (P4)	3.92	160	> 320	> 640	1280	> 5120
T491	YF/WN_{FVR} (P4)	3.92	80	320	160	2560	> 5120
GMT			**80**	**381**	**190**	**1280**	**5120**

a GMT, geometric mean titer; where the endpoint was not determined, the assay limit titer was used in the calculation (e.g., > 640 taken as 640 and <40 taken as 40); b NT = not tested; c animal was euthanized after developing West Nile infection associated symptoms.

Table 8

Viremia (log_{10} pfu) post vaccination; rhesus monkeys were inoculated by the subcutaneous route with YF 17D or YF/WN vaccine candidates as specified.

Monkey	Vaccine (passage level)	Dose Log_{10} PFU	Days post inoculation *										Mean peak titer ± SD	Mean duration (days)
			1	2	3	4	5	6	7	8	9	10		
M017	YF 17D	4.49	0	**1.0**[a]	**2.1**	**2.9**	**2.4**	0	0	0	0	0		
B101	YF 17D	4.49	0	**1.6**	**2.0**	**1.9**	0	0	0	0	0	0	2.4 ± 0.5	3.5
R286	YF 17D	4.49	0	**1.8**	**2.8**	**2.6**	0	0	0	0	0	0		
T081	YF 17D	4.49	**1.3**	**1.0**	**1.5**	**2.0**	0	0	0	0	0	0		
N313	$YF/WN_{107}F$ (P2)	4.19	**1.6**	**2.0**	**1.0**	**1.3**	**1.0**	0	0	0	0	0		
P367	$YF/WN_{107}F$ (P2)	4.19	0	**1.7**	**1.6**	**1.8**	**2.3**	**1.6**	0	0	**1.3**	0	2.2 ± 0.2	5
T087	$YF/WN_{107}F$ (P2)	4.19	**2.3**	**2.3**	**1.3**	**1.3**	0	0	0	0	0	0		
AE81	$YF/WN_{107}F$ (P2)	4.19	**2.3**	**2.1**	**1.6**	**1.3**	0	0	0	**1.0**	0	0		
R918	$YF/WN_{316}V_{440}R$ (P3)	4.0	0	**2.0**	**1.5**	**1.7**	0	0	0	0	0	0		
N577	$YF/WN_{316}V_{440}R$ (P3)	4.0	**1.0**	**1.9**	**1.5**	**1.0**	0	**1.0**	0	0	0	0		
M233	$YF/WN_{316}V_{440}R$ (P3)	4.0	0	0	**1.0**	**1.0**	0	0	0	**1.0**	**1.6**	**1.8**	1.8 ± 0.2	3.5
T757	$YF/WN_{316}V_{440}R$ (P3)	4.0	0	0	0	**1.6**	0	0	0	0	0	0		
J729	YF/WN_{FVR} (P4)	3.92	**1.0**	0	0	**1.0**	**1.3**	**1.0**	0	**1.0**	0	**1.0**		
T445	YF/WN_{FVR} (P4)	3.92	**1.0**	**1.6**	**1.5**	0	0	**1.0**	0	0	0	**1.0**	1.4 ± 0.2	4.5
T086	YF/WN_{FVR} (P4)	3.92	**1.0**	0	**1.3**	**1.3**	0	0	0	0	0	0		
T491	YF/WN_{FVR} (P4)	3.92	0	**1.5**	0	**1.0**	0	0	**1.0**	0	**1.0**	0		

* No virus was detected in the assay at day 0 (pre-inoculation)

[a] Assay lower limit

What is claimed is: